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LONG-TERM EFFECT OF OUABAIN AND SODIUM PUMP INHIBITION ON A NEURONAL MEMBRANE

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SUMMARY

1. The long-term effects of ouabain on the membrane potential of the *Anisodoris* giant neurone (G cell) were examined in cells maintained for periods of up to 15 hr at 11–13° C.

2. In the presence of ouabain (5×10^{-4} M), the membrane potential depolarized to a constant level for 1–4 hr, then hyperpolarized for 5–7 hr after which it gradually depolarized again.

3. During the hyperpolarizing phase, after 6–8 hr in ouabain, $[K]_i$ fell approximately 50 %, $[Na]_i$ increased 50–100 % and the P_{Na}/P_K ratio decreased to 25 % of its initial value.

4. After 8 hr in ouabain the membrane conductance increased two- to fourfold. This increase was independent of temperature and membrane rectification.

5. The K permeability (P_K) was calculated from the constant field equation, and showed a fourfold increase after long-term treatment with ouabain. This rise in P_K probably underlies the membrane hyperpolarization and the decrease in the P_{Na}/P_K ratio.

6. It is suggested that inhibition of the Na^+ pump with ouabain causes a gradual rise in $[Na]_i$ which secondarily leads to Ca^{2+} uptake, an increase in $[Ca]_i$, and thereby an increase in P_K.

INTRODUCTION

There is reasonably good evidence that the cardiac glycosides block the active transport of Na^+ and K^+ in a variety of cells without directly affecting passive membrane permeabilities (Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969*b*; Garrahan, 1970; Marmor, 1971*b*). In nerve cells, where a component of the Na^+–K^+ exchange pump is electrogenic, inhibition of the pump with the cardiac glycoside ouabain depolarizes the membrane, increases the probability of spontaneous action

potentials, and leads secondarily to an increase in intracellular Na^+ and a decrease in intracellular K^+. An increase in internal Na^+ enhances the possibility that intracellular Na^+ can exchange with extracellular Ca^{2+} (Baker, 1972*a*), an effect which might gradually alter membrane permeabilities (Glynn, 1964). Furthermore, if ouabain penetrates the plasma membrane (see Baker & Willis, 1972), and many low molecular weight molecules do if given sufficient time, then it could eventually affect active transport across intracellular membranes as well. These possible long-term effects of ouabain on cell behaviour have not been studied directly.

We have previously shown that the membrane potential of the giant gastroesophageal neurone (G cell) of the marine mollusc, *Anisodoris nobilis*, is dependent upon both ionic diffusion potentials and an electrogenic Na^+–K^+ exchange pump (Gorman & Marmor, 1970*a*). In addition, our results show that inhibition of the pump by ouabain, and the subsequent depolarization of membrane potential, occur without immediate changes in either membrane permeability or in the concentration gradient for K^+ ions across the membrane (Gorman & Marmor, 1970*b*; Marmor, 1971*b*). For these reasons the G cell is a convenient neurone to use for the investigation of indirect and long-term effects of ouabain on cell function. The purpose of this paper is to show that in the presence of ouabain the G cell membrane potential can be maintained for extended periods of time, but that long-term exposure to ouabain causes a significant increase in K permeability.

METHODS

All experiments were performed on the G cell of *Anisodoris nobilis*. The techniques used and the notation for ions were identical to those described in the preceding paper (Gorman & Marmor, 1974). The current–voltage relations were generated by biphasic current clamps (see Marmor, 1971*a*). Ouabain (Mann Research Labs, N.Y.) was dissolved directly in artificial sea-water (ASW). A concentration of 5×10^{-4} M ouabain was used in all experiments.

RESULTS

The resting potential during prolonged exposure to ouabain

In the previous paper (Gorman & Marmor, 1974) we showed that the G cell could be successfully maintained *in vitro*, with intracellular recording electrodes in place, for at least 36 hr. In the present experiments recordings were made in the presence of ouabain at 11–13° C for periods of up to 15 hr (Fig. 1). When ouabain was added to the bathing medium, the cell depolarized because electrogenic Na^+ transport was blocked. For 1–4 hr the resting potential remained stable, often with the cell spontaneously discharging at rates of one action potential per 2–3 seconds as a result of the depolarization. After this period, however, the discharge ceased and

the membrane gradually hyperpolarized as shown in Fig. 1. The hyperpolarization was sustained for 5–7 hr, after which a gradual depolarization was observed.

The hyperpolarization which occurred after 1–4 hr in ouabain was unexpected, since inhibition of the Na^+ pump should cause the cell to gain Na^+, lose K^+, and thus depolarize. The hyperpolarization might be due to the cessation of cell discharge, assuming that some mechanisms other than

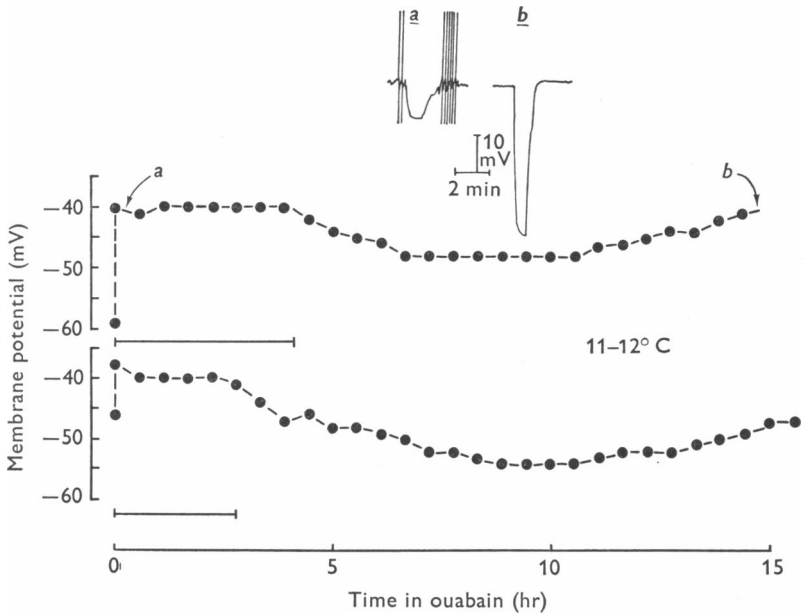

Fig. 1. Effect of ouabain on membrane potential. Data from two different experiments. The initial depolarization from the blocking of the Na^+ pump is shown at time zero. The lines under the first part of each plot indicate the period during which spontaneous firing occurred. The inset above the top plot shows the cell's response to a brief removal of $[K]_o$ (indicated by a bar) at the beginning and end of the experiment (arrows *a* and *b*).

the Na^+–K^+ exchange pump exist to help maintain internal ionic concentrations. Alternatively, long-term changes in membrane permeabilities could account for the hyperpolarization. The inset in Fig. 1 shows the response of the membrane to a brief removal of $[K]_o$ at the start and at the end of 14 hr of treatment with ouabain. The response increased markedly (more than threefold) suggesting that the concentration gradient for K^+, or the permeability characteristics of the membrane, or both, must have changed during this interval.

Decrease in $[K]_i$ *and* P_{Na}/P_K

At any constant temperature and under conditions which block Na^+ pump activity, the G cell membrane potential can be predicted by an approximate constant field type equation simplified to include only terms for Na^+ and K^+ and rewritten in exponential form (Moreton, 1968; Gorman & Marmor, 1970*a*)

$$e^{VF/RT} = \frac{[K]_o + P_{Na}/P_K[Na]_o}{[K]_i}. \quad (1)$$

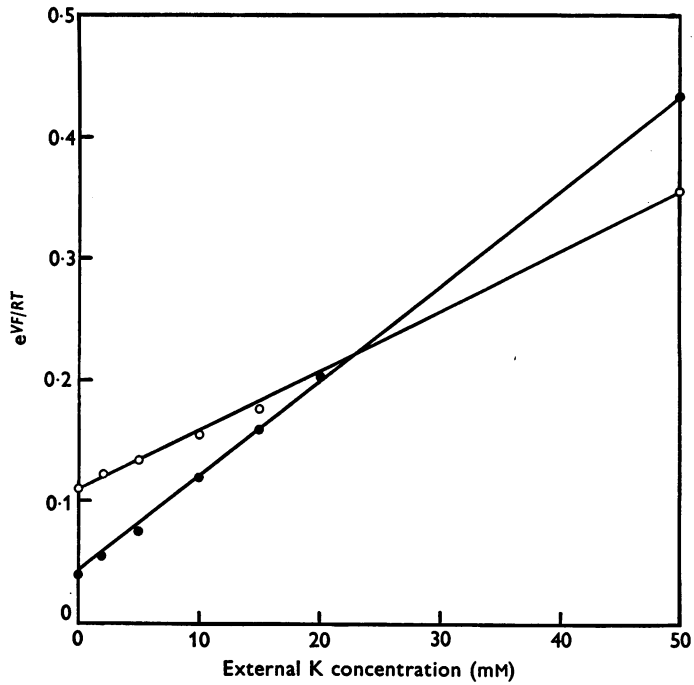

Fig. 2. The relationship between $e^{VF/RT}$ and $[K]_o$ at 3° C before (○—○) and after (●—●) 8 hr immersion in ouabain at 12° C. Data from the same cell. Note the change in the slope and y-intercept of the straight lines drawn by eye through the experimental points (see text for further discussion).

Where P_{Na}/P_K represents the membrane permeability ratio for Na^+ to K^+ ions, the subscripts denote the internal (i) and the external (o) concentrations of these ions and R, T and F have their usual meaning.

Fig. 2 shows the result of a typical experiment in which membrane potential was measured in different $[K]_o$ before and after long-term treatment with ouabain at 12° C. Since the 'before' and 'after' conditions are not directly comparable at 12° C (because of the electrogenic Na pump)

both sets of measurements were made at a cold temperature ($< 5°$ C) which blocks the pump. The cell was cooled briefly for testing with different external K^+ concentrations before warming to 12° C and administering ouabain; after 8 hr of exposure to ouabain the cell was cooled again and re-tested. The data are plotted as $e^{VF/RT}$ *vs.* $[K]_o$, and in agreement with eqn (1), the points fall on straight lines so that $[K]_i$ (the slope) and P_{Na}/P_K (the y-intercept) can be calculated. $[K]_i$ fell from 204 mM to a value of 126·6 mM after ouabain treatment, whereas P_{Na}/P_K decreased

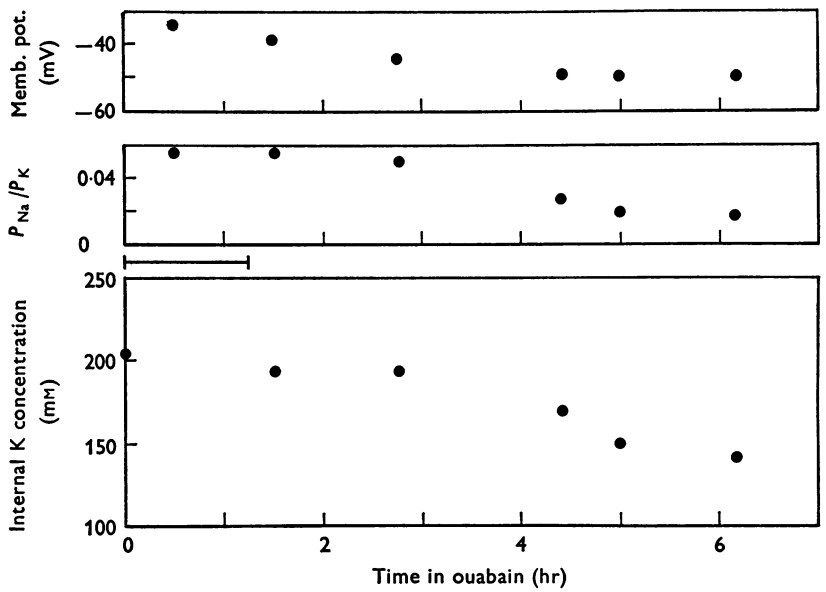

Fig. 3. Change in membrane potential, P_{Na}/P_K and $[K]_i$ with time in the presence of ouabain. Data are shown for the same cell at 12° C over a period of 6 hr. The line under the beginning of the plot indicates the period during which spontaneous firing occurred.

from 0·047 before treatment to 0·012 after 8 hr in ouabain. Similar changes were present when data obtained at 12° C immediately after entry into ouabain were compared to those obtained at 12° C after long-term treatment (see Fig. 3) indicating that the changes are not dependent upon temperature.

Fig. 3 shows an example of a cell maintained in ouabain at 12° C in which changes in membrane potential, $[K]_i$ and P_{Na}/P_K were measured at various intervals over a period of 6 hr. The data were obtained at the same temperature (12° C) and show that P_{Na}/P_K and $[K]_i$ change with time. The hyperpolarization of membrane potential presumably reflects the net

difference between the depolarization expected for a decrease in $[K]_i$ and the hyperpolarization expected for a decrease in P_{Na}/P_K.

Increase in $[Na]_i$

Unless there are other means for maintaining a low internal Na^+ besides the Na^+ pump, $[Na]_i$ should progressively increase with time when the G cell is left in ouabain. We have observed in many cells that the soma action potential overshoots zero membrane potential by 40–60 mV and is abolished by complete replacement of $[Na]_o$ with sucrose, Tris or choline (plus D-tubocurare). To determine whether the action potential peak could be used as an index of $[Na]_i$, action potentials were examined in two cells over a range of $[Na]_o$, using Tris as a replacement for Na^+. The overshoot was linearly related to log $[Na]_o$ between 120 and 480 mM-Na^+, suggesting that the Nernst equation does give a reasonable estimate of $[Na]_i$ (although there is undoubtedly some mixture of Na and K permeabilities at the peak of the action potential).

In two experiments the action potential was monitored for approximately 8 hr after entry into ouabain. In both cases (Fig. 4) the estimated value for $[Na]_i$ (inferred from the action potential peak) was unchanged by the initial depolarization produced by ouabain, but increased rapidly thereafter once the cell began to spontaneously discharge. This increase continued at a slower rate after the discharge ceased and the cell began to hyperpolarize. Over 8 hr in ouabain, $[Na]_i$ was estimated to rise to between 1·5 and 2 times its initial value.

Increase in K permeability

The finding that the P_{Na}/P_K ratio decreased during exposure to ouabain (Fig. 2) could be explained by either a decrease in P_{Na} or an increase in P_K and the results presented to this point do not discriminate between these two possibilities. To resolve this question, the slope conductance of the G cell was measured at different membrane potential values at 11° C before ouabain, immediately after ouabain, and after 8 hr immersion in ouabain (Fig. 5). The addition of ouabain caused a prompt depolarization but did not alter the conductance at comparable membrane potentials. After 8 hr in ouabain, however, there was a two- to fourfold increase in total membrane conductance at all membrane potential levels. Measurements of conductance at various intervals after immersion in ouabain show that increase in conductance is associated temporally with the hyperpolarization.

Since the G cell conductance is primarily carried by K^+ ions, these results suggest that P_K increases during exposure to ouabain. To confirm this hypothesis, current–voltage plots were studied before and after long-term exposure to ouabain, at 3° C where the constant field equation could

be fit to the curves and used to calculate P_K (see Marmor, 1971*a*). Fig. 6 shows current–voltage plots at 11 and 3° C, before and after ouabain, to show that whereas cooling eliminated the inward-going ('anomalous') rectification which is present at 11° C, it did not mask the striking change in conductance produced by long-term exposure to ouabain. The smooth curves through the points at 3° C represent a fit to the constant field equation using the parameters in the caption. P_K, calculated from those

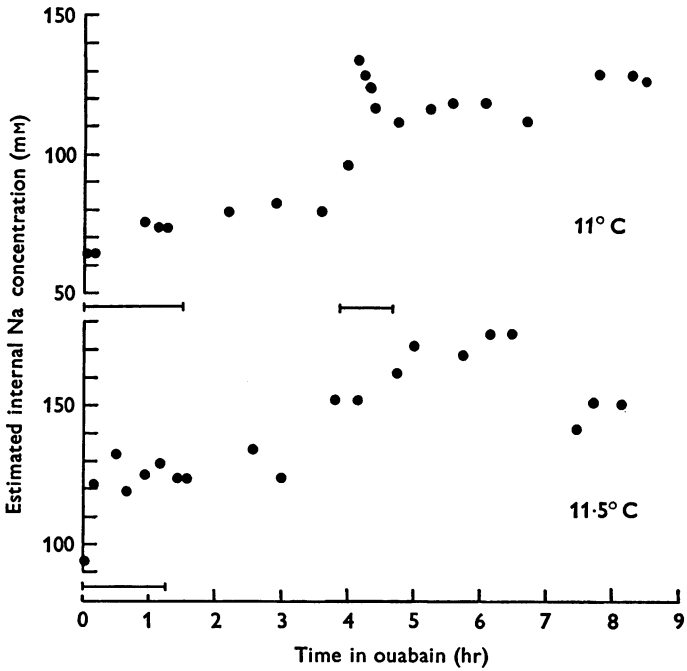

Fig. 4. Change in $[Na]_i$ with time in the presence of ouabain. Data from two experiments. The experimental values were estimated from the peak of the action potential ($E_{A.P.\ peak}$) using the Nernst equation for Na^+ ions where $E_{A.P.\ peak} \approx E_{Na} = RT/F \ln [Na]_o/[Na]_i$ (see text). The lines under the plots indicate periods where spontaneous firing occurred.

curves, rose from $2{\cdot}2 \times 10^{-8}$ cm/sec before ouabain to $9{\cdot}6 \times 10^{-8}$ cm/sec after ouabain. Similar values were found in two other cells before and after ouabain. Thus, ouabain exposure induced roughly a fourfold *increase* in P_K while producing little or no change in P_{Na} (since we noted above that P_{Na}/P_K *decreased* about fourfold after ouabain exposure). Note that even the increased values for P_K are considerably smaller than the absolute values reported in other neurones such as the squid axon ($1{\cdot}8 \times 10^{-6}$ cm/sec, Hodgkin & Katz, 1949).

Prolonged exposure to cold

Although the primary purpose of these experiments was to study the effects of ouabain, one cell was maintained for 17 hr at 0° C to compare an alternate method of pump inhibition. During the first 14 hr a mild hyperpolarization occurred, as with ouabain, followed by a slow depolarization. However, test pulses of 0 K ASW at the onset and termination of the cold exposure only showed a change from 15 mV hyperpolarization

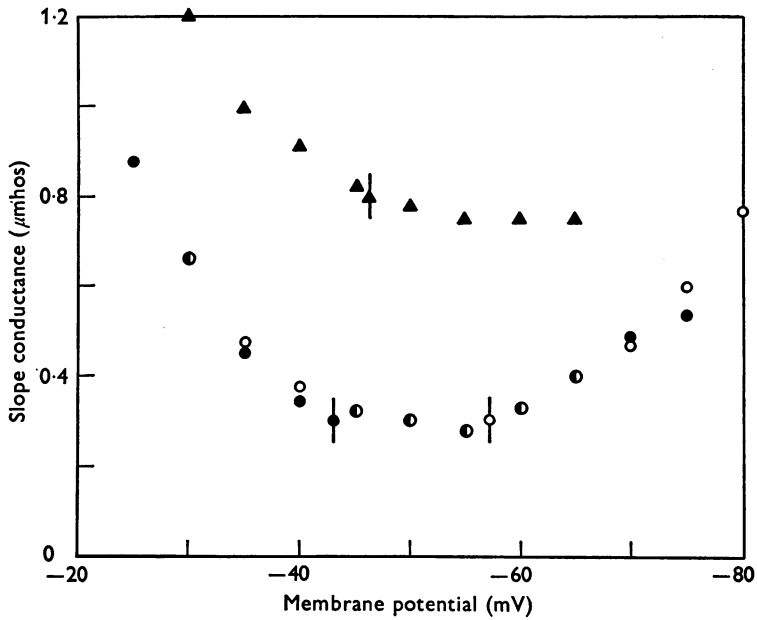

Fig. 5. Effect of ouabain on membrane slope conductance (dI/dV). Data are shown for the same cell at 11° C before (○), immediately after (●) and 8 hr after (▲) immersion in ouabain. The half-filled circles represent data points where the open and filled circles coincide. The resting membrane potential is indicated in each case by a vertical line.

to 18 mV hyperpolarization. It is clear that the G cell can maintain potential for extended periods in the cold; but it appears that no dramatic changes in K permeability occur within 17 hr judging by the 0 K ASW response. On the other hand, the mild hyperpolarization of resting potential and the small increase in the 0 K ASW response, would be consistent with the phenomena seen in ouabain if its time course were much slower in the cold.

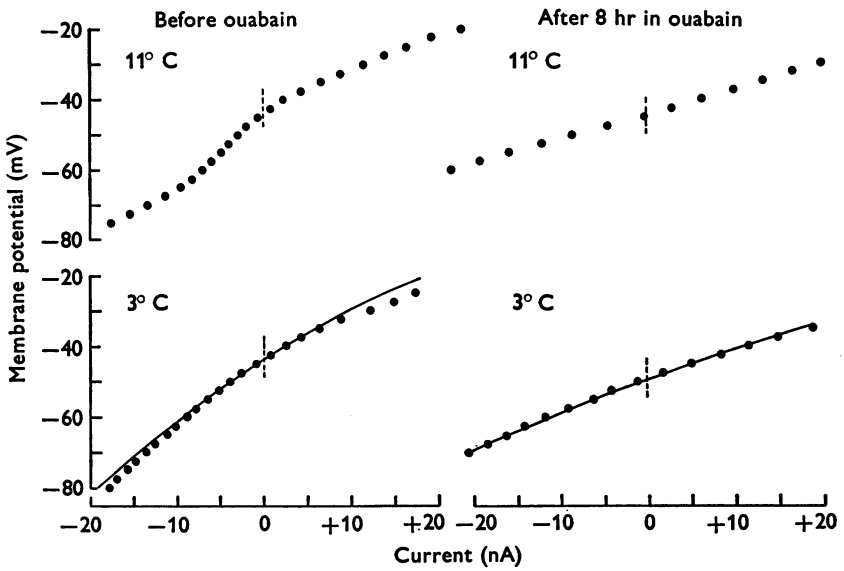

Fig. 6. Effect of ouabain on current–voltage relation. Data shown for the same cell at two temperatures before and after 8 hr immersion in ouabain at 11° C. The dashed lines indicate resting potential (zero current) in each case. The continuous curves through the two plots in the cold were drawn from the constant field equation, where

$$I = \frac{VF^2P_K[K]_o + P_{Na}/P_K[Na]_o - [K]_i e^{VF/RT}}{RT(1-e^{VF/RT})},$$

for the following parameters: for both curves, $[K]_o$ = 10 mM, $[Na]_o$ = 480 mM and T = 215° K; for the left curve, $[K]_i$ = 204 mM, P_{Na}/P_K = 0·047 and $P_K = 2{\cdot}2 \times 10^{-8}$ cm.sec^{-1}; for the right curve, $[K]_i$ = 127 mM, P_{Na}/P_K = 0·012 and $P_K = 9{\cdot}6 \times 10^{-8}$ cm.sec^{-1}.

DISCUSSION

Our results provide the first clear demonstration that ouabain treatment, in addition to inhibiting the Na^+–K^+ exchange pump, can alter membrane permeability. However, they also demonstrate that this effect on permeability is delayed, and probably indirect. Our data indicate that an increase of P_K presumably accounts for the associated observations that the cell hyperpolarizes, $[K]_i$ and P_{Na}/P_K decrease, and membrane conductance increases. P_{Na} does not seem to be affected significantly by ouabain exposure, and the G cell is relatively impermeable to Cl^- (Gorman & Marmor, 1970*a*).

Previous data from the G cell (Marmor, 1971*b*), the squid axon (Baker *et al.* 1969*b*) and red blood cells (see Garrahan, 1970) indicate that ouabain has no direct effect on membrane permeability. None of our present findings

contradict this conclusion. Quite the contrary: the hyperpolarization, the decrease in P_{Na}/P_K and the conductance increase are apparent only after 1–4 hr in ouabain. It is conceivable, of course, that earlier changes in permeability occur which went undetected because of inadequacies in our measurement technique. However, the progressive changes in membrane properties seem more suggestive that an accumulation of some intracellular product occurs in the presence of ouabain and secondarily alters P_K.

Baker & Willis (1972) have presented evidence which suggests that ouabain slowly accumulates inside cells when used in high concentration. We cannot rule out the possibility that an increase in intracellular ouabain affects P_K either directly or indirectly. However, ouabain has no effect on the Na^+–K^+ pump of the squid axon when placed inside (Caldwell & Keynes, 1959) and is ineffective against other transport systems in the plasma membrane (Russell & Brown, 1972*a*). An alternative explanation is that the change in P_K is related to the change in the absolute levels of $[Na]_i$ and $[K]_i$. Our results show that ouabain induces a slow fall in $[K]_i$ and a rise in $[Na]_i$ and both of these changes occur in parallel with the change in the G cell's membrane properties. In squid axon (Baker, Blaustein, Hodgkin & Steinhardt, 1969*a*), a rise in $[Na]_i$ increases the influx of Ca^{2+} ions and there is a growing body of evidence that the passive permeability of the plasma membrane is dependent on the intracellular concentration of free Ca ions (see Baker, 1972*b*). Any increase in $[Ca]_i$ could in theory be compensated by Ca^{2+} accumulation in the mitochondria (Lehninger, Carafoli & Rossi, 1967) except that the mitochondrial uptake of Ca^{2+} appears to be inversely related to the absolute level of $[Na]_i$ (Dransfeld, Greef, Hess & Schorn, 1967). An increase in $[Ca]_i$ results in a specific increase in P_K in red blood cells (Lew, 1970; Romero & Whittam, 1971), in liver cells (Van Rossum, 1970), in *Aplysia* neurones (Meech, 1972) and in mammalian motoneurones (Krnjević & Lisiewicz, 1972). Moreover, in *Aplysia* neurones (Carpenter, Snover & Barker, 1971), in *Pecten* photoreceptor cells (Gorman & McReynolds, 1974) and in mammalian cortical neurones (Godfraind, Kwamura, Krnjević & Pumain, 1971) metabolic inhibitors which block the production of ATP induce a hyperpolarization and increase in K^+ conductance which may be mediated by an increase in $[Ca]_i$.

The suggestion that a rise in $[Ca]_i$ provides a link between the long-term effects of ouabain and changes in membrane permeability is not completely new. Similar suggestions have been made to explain the effect of very low concentrations of ouabain on the contractile force of heart muscle, i.e. the positive inotropic effect (Glynn, 1964). There may also be important differences between cells, e.g. in some photoreceptors (Lisman & Brown, 1972) an increase in $[Ca]_i$ apparently reduces P_{Na} rather than increasing P_K.

If the effects of ouabain on P_K are solely mediated through inhibition of the Na^+ pump and the resultant changes in internal ionic concentrations, then other methods of pump inhibition should produce the same net result. Russell & Brown (1972*b*) have shown that the membrane potential of *Aplysia* neurones hyperpolarizes during long-term exposure to both ouabain and cold despite a continuing decrease in $[K]_I$. Our data in cold is limited to one experiment and does not resolve this question. Exposure to cold did not appear to raise P_K significantly in 17 hr, but a small rise did occur judging by the response to removing $[K]_o$. It is plausible that cold should affect internal ionic concentrations much more slowly than ouabain since the cell does not show spontaneous action potentials in the cold and P_{Na} is much smaller (Gorman & Marmor, 1970*b*; Marmor, 1971*b*).

A corollary to the paragraph above is the question of how the cell maintains potential in the absence of an active Na^+–K^+ exchange pump. For 1–4 hr in ouabain, and for 15 hr in the cold, the G cell maintains a relatively steady membrane potential. This stability may simply reflect the low permeability characteristics of the G cell membrane, especially in the cold. The G cell belongs to a class of cell with a large surface-to-volume ratio (Gorman & Mirolli, 1972; Mirolli & Talbott, 1972), and there are obvious adaptive advantages to limiting ionic movement under resting conditions. Alternatively, other ion transport mechanisms such as Na-Ca exchange (Baker, 1972*b*) may be less sensitive to temperature and may help in maintaining homoeostasis.

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